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In vitro RNA editing in plant mitochondria does not require added energy

Mizuki Takenaka[a,*], Daniil Verbitskiy[a], Johannes A. van der Merwe[a], Anja Zehrmann[a], Uwe Plessmann[a,b], Henning Urlaub[a,b], Axel Brennicke[a]

[a] *Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany*
[b] *Max Planck Institute for Biophysical Chemistry, Bioanalytical Mass Spectrometry Group, Am Fassberg 11, 37077 Göttingen, Germany*

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Abstract **RNA editing in flowering plant mitochondria is investigated by in vitro assays. These cauliflower mitochondrial lysates require added NTP or dNTP. We have now resolved the reason for this requirement to be the inhibition of the RNA binding activity of the glutamate dehydrogenases (GDH). Both GDH1 and GDH2 were identified in RNA–protein cross-links. The inhibition of in vitro RNA editing by GDH is confirmed by the ability of the GDH-specific herbicide phosphinothricin to substitute for NTP. NADH and NADPH, but not NAD or NADP, can also replace NTP, suggesting that the NAD(P)H-binding-pocket configuration of the GDH contacts the RNA. RNA editing in plant mitochondria is thus intrinsically independent of added energy in the form of NTP.**

Keywords: RNA editing; Plant mitochondria; Glutamate dehydrogenase; RNA-binding; In vitro RNA editing; Cauliflower

1. Introduction

In plants RNA molecules are in both organelles altered by RNA editing. In plastids of flowering plants about 35 and in mitochondria about 400 selected cytosines are changed to uridines. The direct biochemical effect of RNA editing in these plants is thus a site-specific deamination. Despite years of investigation neither the reaction mechanism nor the enzymes involved have been identified. Several clues on – or rather conditions of – the biochemistry of the reaction have emerged mostly from in vitro analyses of plastid and mitochondrial lysates, respectively. The first in vitro assays of plant mitochondrial lysates suggested that the sugar-phosphate bonds of the affected RNA molecule are not disrupted in the polynucleotide chain [1]. This observation excludes insertional editing which would excise and exchange either the nucleotide or the base. Thus either direct deamination or transamination are the most likely mechanisms. The first reaction would per se require no added energy, while for the latter, the transamination reaction, additional molecules of higher molecular energy would most likely be involved.

For mitochondrial lysates a strict requirement for added ATP has been observed [2]. Similarly in plastids additional ATP has been reported to be required in most lysates [3–5], while in some residual activity can be seen without any added ATP [6,7]. Surprisingly the added ATP can be substituted to full effect by some or all of the other NTPs and even dNTPs [2,6,7]. While all of these molecules are virtually interchangable in mitochondrial lysates, they vary in their effect in plastid extracts [6,7]. In both organelles at least one of the dNTPs is as effective as ATP, suggesting that one of the few enzymes accepting either triphosphate is involved. One group of such enzymes is a class of RNA helicases and their participation has consequently been proposed [2].

We have now investigated the requirement for nucleotide triphosphates in detail and come to the surprising conclusion that this dependence is at least in mitochondria almost entirely explained by the behaviour of the enzyme glutamate dehydrogenase (GDH).

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Cauliflower mitochondria were purified by differential centrifugation steps and a Percoll gradient as described [2,8]. Isolated mitochondria were lysed, the lysate was cleared and the supernatant was recovered and dialyzed as detailed previously [2,8].

2.2. RNA substrates

DNA clones were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as described [2,8]. Substrate RNAs containing vector sequences at the 5′- and 3′-ends of the mitochondrial insert sequences were synthesized from the T7 RNA polymerase promoter in the linearized template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the in vitro assay [9,10]. The ^{32}P-labelled RNA templates for the gel shift assays were obtained by direct incorporation of labelled ATP into the RNA during run-off transcription from the DNA template.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [8,11]. After incubation, substrate sequences were amplified by RT-PCR, the upstream primer being labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase, Trevigen). The TDG treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (GE Healthcare).

*Corresponding author. Fax: +49 731 502 2626.
E-mail addresses: mizuki.takenaka@uni-ulm.de (M. Takenaka), daniil.verbitskiy@uni-ulm.de (D. Verbitskiy), dre.van-der-merwe@uni-ul.de (J.A. van der Merwe), anja.zehrmann@uni-ulm.de (A. Zehrmann), uplessm@gwdg.de (U. Plessmann), henning.urlaub@mpi-bpc.mpg.de (H. Urlaub), mo.bo@uni-ulm.de (A. Brennicke).

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2.4. RNA binding proteins

Proteins in the mitochondrial lysate binding to RNA were analysed by incubating 1 pmol RNA template for 30 min in the reaction mixture. In the template RNA every uridine was labeled with ^{32}P by in vitro transcription without adding cold UTP. The proteins in contact with the RNA were irradiated with UV light for 20 min (Stratalinker 1800, stratagene). This step was followed by digestion of the RNA with RNase A at 37 °C for 30 min. Protein samples were dissolved in buffer and separated by SDS–PAGE on a 10% gel. Labelled proteins were visualized with a Bioimaging Analyzer BAS-3000 (Fuji Photo Film Co).

2.5. Protein identification

RNA editing templates were labelled with biotin (biomers GmbH) and 500 pmol were incubated with 100 μl of mitochondrial lysate in a total volume of 200 μl under standard in vitro RNA editing conditions in the presence of 1 mg total yeast RNA [2]. The proteins in contact with the RNA were cross-linked by UV irradiation for 20′ (UV Stratalinker 1800, Stratagene). The biotin-labelled RNA–protein complexes were bound to streptavidine sepharose™ High performance (GE healthcare). Unbound proteins were washed off by six rinses in spin columns with 400 μl wash buffer each (30 mM HEPES-KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30 mM ammonium acetate, 10% glycerol). The sepharose beads were treated with RNase A to release the RNA-bound proteins, which were collected with wash buffer. Proteins with their bound residual RNA fragments were analysed by PAGE and/or were (directly) identified by liquid chromatography coupled (LC) electrospray ionization (ESI) tandem mass spectrometry (MSMS). Proteins cross-linked to RNA were digested within the gel using trypsine according to Shevchenko et al. [12]. For digestion of proteins in solution after extraction from the beads, the samples are denatured in 4 M urea and 2 M thiourea, diluted to a final concentration of $\leqslant$1 M urea and incubated with 2 μg trypsine overnight at room temperature. Peptides are desalted and separated by nanoLC equipped with a pre-column working in backflush and directly analyzed by ESI MSMS in a Q-ToF (Q-ToF ultima, Waters) or a hybrid triple quadrupole/linear iontrap mass spectrometer (4000 QTrap, ABI) under standard conditions. Fragment spectra of peptides are searched against the NCBInr database using Mascot as search engine. Matches with resulting protein identification were usually obtained against the *Arabidopsis thaliana* sequences, which is phylogenetically closely related to the here analysed cauliflower (*Brassica oleracea*). Since different species are compared, similarities/differences in the respective orthologous protein sequences additionally influence the generated scores.

3. Results and discussion

3.1. NTP as well as dNTP support the in vitro RNA editing reaction

The strict requirement for added NTP in the in vitro RNA editing reaction is documented in Fig. 1. No RNA editing product with uridine in the relevant position of RNA editing is detectable if no NTP is added. Addition of ATP at the optimal concentration of 15 mM yields about 2–5% C to U converted nucleotide, the amount depending upon the lysate, the source of the mitochondria and the template used. The positive effect of ATP can be fully recovered by substitution with CTP, shown as example here (Fig. 1). Any of the NTPs or dNTPs can replace ATP and will yield comparable editing rates [2]. Since in plastid as in mitochondrial in vitro systems NTPs and dNTPs can substitute for ATP, analogous factors may be responsible for the in vitro ATP requirement in both organelles.

3.2. Influence of the added NTP on template RNA–protein interactions

To investigate the protein moieties affected by the added NTP/dNTP in their ability to contact and bind to the RNA

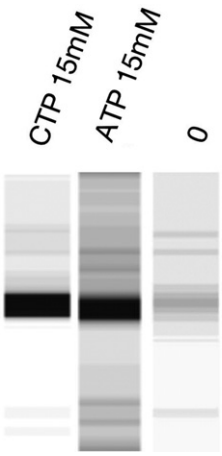

Fig. 1. In vitro RNA editing of an *atp4* mRNA template requires the addition of ATP or CTP. Similar to the in vitro editing of an *atp9* RNA [2], dNTP-nucleotides can substitute for the NTP in this reaction (data not shown).

substrate molecules, we performed gel shift experiments with radioactively labelled RNA editing substrates (data not shown). Comparisons of the protein patterns cross-linked with the template RNA in the presence or absence of ATP on PAGE-gels and by more sensitive mass spectroscopy (MS)-analyses did not reveal any qualitative differences. The overall quantity of the proteins labelled with the cross-linked RNA, however, changes dramatically with the presence or absence of NTP: Without added ATP the same proteins are labelled much stronger (Fig. 2). This suggests that addition of ATP disturbs all protein–RNA interactions non-specifically.

3.3. Identification of proteins bound by the RNA editing template

Since the gel shift and UV cross-linking experiments (Fig. 2) did not yield detectable proteins differentially affected by NTP in their affinity to the RNA editing template, we employed a more sensitive affinity purification scheme to identify respective polypeptides. This procedure achieved an enrichment of the proteins bound to the editing template RNA (Fig. 3). The Coomasie-stained SDS–PAGE patterns of proteins retained by the RNA template in the presence or absence of ATP, however, again showed only little qualitative difference and no clear candidate protein could be assigned by its ATP-dependent affinity to the template in this gel analysis (data not shown).

Because the ATP-dependence of the in vitro reaction may not manifest in abundant specific RNA–protein interactions and the PAGE analysis may not be sensitive enough, we proceeded to analyse the set of proteins interacting with the RNA directly by the much more sensitive MS without prior gel separation. The proteins bound by the RNA editing template were hydrolyzed and peptides were identified by liquid chromatography (LC) coupled tandem MS (MSMS) analysis. To identify the proteins, the obtained fragment spectra were searched against all entries in the NCBInr database. Since cauliflower and *A. thaliana*, which latter is completely sequenced, are closely related and the protein sequences of many household enzymes are to a large extent identical, we should be able to identify cauliflower proteins through their

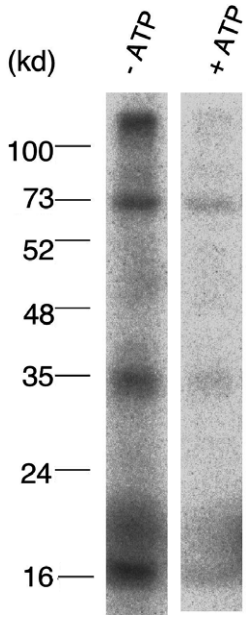

Fig. 2. Similar proteins are cross-linked to radiolabelled RNA in the presence or absence of ATP, but ATP reduces the overall amount of the unspecifically bound proteins. Proteins contained in the mitochondrial lysate from cauliflower inflorescences used for in vitro RNA editing assays were UV-cross-linked to ^{32}P labelled *atp4* RNA in the absence (−ATP) or presence (+ATP) of 15 mM added ATP. The RNA template was digested with RNase A and proteins were separated by SDS–PAGE in a 10% gel. Sizes of marker proteins are indicated in kDa on the side of the autoradiogramme of the dried gel.

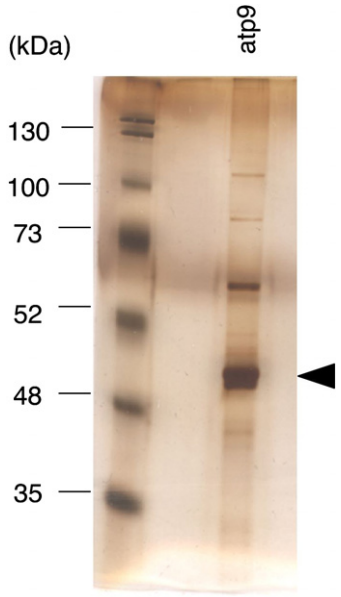

Fig. 3. Cauliflower proteins binding to the atp9 RNA editing template were purified by cross-linking to the RNA. The *atp9* RNA editing template contains 40 nucleotides upstream and 10 nucleotides downstream of the first editing site. Proteins were spread on an analytical PAGE-gel and silver-stained. The arrow indicates the position of the GDH as determined from the molecular weight of the *A. thaliana* protein(s). It is assumed that the cauliflower protein analysed here has a comparable molecular weight.

orthologues in *A. thaliana*. The proteins contained in the total complement enriched by the affinity purification procedure and identified by conserved peptide sequences indeed yielded a list of mitochondrial proteins from *A. thaliana* (Table 1).

Within this list we focussed on proteins which might be involved in a deaminase or transaminase reaction. Among the general mitochondrial proteins such as heat shock proteins, malate dehydrogenase and such, some of the more prominent proteins were representatives of the glutamate dehydrogenase group of proteins, i.e. GDH1 and GDH2 (Table 1) [13]. GDH can catalyze amination as well as deamination reactions depending on the presence and concentration of various allosteric and isosteric regulators (Fig. 4). There is thus a (albeit remote) possibility that one or more of these enzymes have been recruited into the RNA editing reaction to catalyse the deamination step from C to U.

Furthermore GDH-proteins have been previously identified as RNA binding proteins [14] and have been investigated particularly with respect to their participation in RNA editing in kinetoplasts of trypanosomes. Simpson and coworkers showed that the preferential binding of GDH to guide RNA is fortuitous and not related to RNA editing [15]. However, in kinetoplasts RNA editing involves the insertion and deletion of specific uridines rather than the deamination events found in mitochondria and plastids of flowering plants.

3.4. Is the GDH involved in the RNA editing reaction?

To investigate a potential participation of the GDH in the C to U deamination reaction, we tested the influence of various cofactors of this enzyme on the in vitro editing assay. The most prominent and essential cofactors of the normal GDH catalyzed reactions are NADH and NADP for the deamination and amination reactions, respectively. NAD, NADP and their respective reduced forms NADH and NADPH were individually added to the in vitro RNA editing reaction. The assay also contained the suboptimal concentration of 10 mM ATP to be able to observe either enhancing or detrimental effects. NADH as well as NADPH stimulated the reaction while the oxidized dinucleotides NAD and NADP did not (data not shown). Since only enhancing effects were detected, the next series of assays investigated the effect of NADH and the other dinucleotides in the absence of ATP to see whether they can substitute at least partially for the mononucleotide. Both NADH and NADPH were able to replace the NTP requirement, while NAD and NADP did not support the in vitro RNA editing reaction (Fig. 5).

3.5. Investigation of the role of the GDH in the in vitro RNA editing assay

This result of NADH and NADPH being able to substitute for NTP suggests that NTP may not be required for the RNA editing reaction per se, but may be required to alleviate the inhibitory effect of the GDH by releasing this protein from the template RNA or by keeping it away a priori. To test this hypothesis that the GDH might be a major inhibitor of the in vitro RNA editing activity, we analysed the effect of a direct blocker of the GDH proteins.

The compound phosphinothricin is such a specific inhibitor of the GDH in plants and binds irreversibly to the active site of the enzyme. Addition of phosphinothricin to the in vitro RNA editing reaction indeed recovered the editing activity in the absence of any added NTP (Fig. 6). This result shows that NTP solely serves to keep the GDH protein(s) away from the

Table 1
Typical protein identification results of an MS analysis after enrichment and UV-cross-linking of cauliflower mitochondrial proteins binding to the *atp9* RNA editing template

Gene identifier	Mass	Score	Queries matched	Name
gi|4210330	116581	839	19	2-oxoglutarate dehydrogenase, E1 subunit
gi|7076784	114147	575	12	2-oxoglutarate dehydrogenase, E1 subunit-like protein
gi|2654210	72330	489	9	Heat shock 70 protein
gi|17939849	63332	435	7	Mitochondrial F1 ATP synthase beta subunit
gi|15238762	44496	324	7	GDH1 (GLUTAMATE DEHYDROGENASE 1); oxidoreductase
gi|520478	39163	258	4	Pyruvate dehydrogenase E1 beta subunit
gi|4210332	49908	226	7	2-oxoglutarate dehydrogenase E2 subunit
gi|415733	62316	210	4	Mitochondrial chaperonin
gi|15240793	44671	191	4	GDH2 (GLUTAMATE DEHYDROGENASE 2); oxidoreductase
gi|710400	43003	134	5	Pyruvate dehydrogenase E1 alpha subunit
gi|6435320	25248	123	3	Nucleoside diphosphate kinase

The orthologous proteins from *A. thaliana* with relevant scores listed here are those expected in plant mitochondria and previously identified in proteome analyses [16].

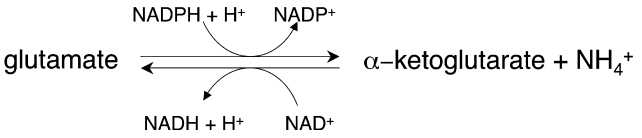

Fig. 4. The reactions catalyzed by the glutamate dehydrogenase include deamination and amination steps. The GDH enzymes bind NAD(P) or NAD(P)H for the activities of amination and deamination, respectively. The direction of the dominant reaction is influenced by the levels of ATP. It is possible that one of these or a similar enzyme has mutated to access also RNA molecules and deaminate specific cytidines.

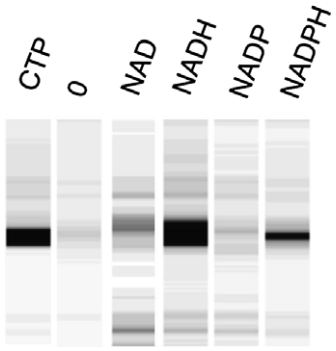

Fig. 5. NADH and NADPH can substitute the requirement for NTP in the in vitro RNA editing reaction. These assays monitor the first editing site in an *atp4* template RNA in which no other site can be edited since the adjacent downstream nucleotides have been altered [9]. The lane marked 0 contains no added (di)nucleotide in the control in vitro incubation. The lanes marked CTP, NADH, NAD, NADP, NADPH contain 15 mM of the respective compounds in the in vitro reaction mix. The gel image shown is the relevant portion of the fluorescent detection of the Cy5 labelled RT-PCR products after TDG treatment, which recognizes the mismatches at the T moieties introduced by in vitro editing.

template and that no added energy is required for the RNA editing reaction per se.

The equivalent capability of NADH and the other reduced dinucleotide NADPH suggests that the site of action of these compounds is the GDH itself. Whether the added NTPs and dNTPs also bind to the GDH remains open.

If the NTPs and dNTPs do not directly interact with the GDH, the NTP/dNTP and the NADH/NADPH/phosphinothricin effects could be achieved by different modes of action: While the first may activate an RNA helicase [2] which removes the inhibiting bound GDH, the latter compounds may have the same final effect by directly blocking the GDH. The ultimate result observed with both sets of added chemicals, namely active in vitro RNA editing, could be the outcome from either mechanism. To decide this question, the effect of added (d)NTPs and NAD(P)H on the RNA binding of the GDH will have to be investigated with the purified GDH enzyme. The observation that all unspecific RNA-binding proteins in a mitochondrial extract are diminished upon addition of ATP to the gel-shift or cross-linking reaction (Fig. 2) suggests that the NTP/dNTP effect is indeed not a direct action on the GDH, but could be rather a separate general RNA-wiping effect, possibly mediated by an RNA helicase.

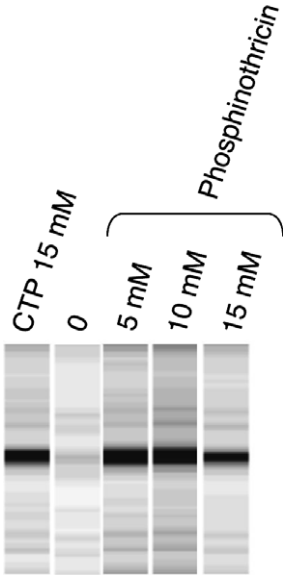

Fig. 6. The GDH-specific inhibitor phosphinothricin can substitute the requirement for NTP in the in vitro RNA editing reaction. All assays monitor the first editing site in an *atp4* template RNA. In the control in vitro incubation no nucleotide was added (lane marked 0). The lanes marked "phosphinothricin" contain this compound but no added NTP or dNTP in the in vitro reaction mix. Only the relevant portion of the gel image is shown.

Nevertheless, the substitution of NTP by phosphinothricin shows that if another, general RNA-clearing enzyme such as a helicase is stimulated by the added NTP/dNTP, the activity of this enzyme is not essential for the actual RNA editing reaction, but at least in vitro mainly serves to remove the inhibitory GDH.

3.6. RNA editing does not require added NTP in vitro

The observed in vitro RNA editing in the absence of added NTP suggests that the actual biochemical reaction does not consume energy. The in vitro observed requirement of added NTPs, dNTPs, NADH or phosphinothricin may not be relevant in the in vivo situation: in the intact mitochondrion, RNA editing as well as splicing and 5′- and 3′-processing most likely occur compartimentalized and in safe distance from the location of the GDH.

In chloroplast extracts in vitro RNA editing is similarly stimulated by or depends on the addition of NTP or dNTP moieties. Here the plastid located NADPH-dependent GDH may play an analogous inhibitory role and corresponding specific inhibitors such as phosphinothricin should be tested to resolve this question. It has been reported however that neither NADH nor NADPH have any effect on the in vitro reaction, which may argue against an analogous role [6]. On the other hand the effects of individual NTP and dNTP identities vary between chloroplast extracts from different plant species [3,6,7].

Of course one cannot formally exclude the presence of low amounts of ATP in the mitochondrial or chloroplast lysates which escape the dialysis step by being bound to larger molecules during the extract preparation. However, direct determination of the ATP content in a plastid lysate showed less than 10 nM ATP to be present, suggesting that the dialysis indeed efficiently removes these small molecules [6].

The correct in vitro RNA editing in a plant mitochondrial lysate in the presence of GDH-inhibiting phosphiniothricin shows that the GDH is not involved in the deamination of C to U although this enzyme regularly catalyzes an analogous reaction and can bind RNA molecules. These conclusions make a direct deamination process by a modified cytidine deaminase or an analogous enzyme more probable than a transaminase reaction, for which a requirement of some additional source of activation energy would be more likely – although energetically not strictly necessary.

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References

[1] Araya, A., Domec, C., Begu, D. and Litvak, S. (1992) An in vitro system for the editing of ATP synthase subunit 9 mRNA using wheat mitochondrial extracts. Proc. Natl. Acad. Sci. USA 89, 1040–1044.
[2] Takenaka, M. and Brennicke, A. (2003) In vitro RNA editing in pea mitochondria requires NTP or dNTP, suggesting involvement of an RNA helicase. J. Biol. Chem. 278, 47526–47533.
[3] Hirose, T. and Sugiura, M. (2001) Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of chloroplast RNA: development of an in vitro RNA editing system. EMBO J. 20, 1144–1152.
[4] Miyamoto, T., Obokata, J. and Sugiura, M. (2002) Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of *cis*-acting elements and *trans*-acting factors involved in RNA editing in tobacco and pea chloroplasts. Mol. Cell. Biol. 22, 6726–6734.
[5] Miyamoto, T., Obokata, J. and Sugiura, M. (2003) A site-specific factor interacts directly with its cognate RNA editing site in chloroplast transcripts. Proc. Natl. Acad. Sci. USA 101, 48–52.
[6] Hegeman, C.E., Hayes, M.L. and Hanson, M.R. (2005) Substrate and cofactor requirements for RNA editing of chloroplast transcripts in Arabidopsis in vitro. Plant J. 42, 124–132.
[7] Nakajima, Y. and Mulligan, M. (2006) Nucleotide specificity of the RNA editing reaction in pea chloroplasts. J. Plant Physiol. 162, 1347–1354.
[8] Neuwirt, J., Takenaka, M., van der Merwe, J.A. and Brennicke, A. (2005) An in vitro RNA editing system from cauliflower mitochondria: editing site recognition can vary between different plant species. RNA 11, 1563–1570.
[9] Verbitskiy, D., Takenaka, M., Neuwirt, J., van der Merwe, J.A. and Brennicke, A. (2006) Partially edited RNAs are intermediates of RNA editing in plant mitochondria. Plant J. 47, 408–416.
[10] van der Merwe, J.A., Takenaka, M., Neuwirt, J., Verbitskiy, D. and Brennicke, A. (2006) RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett. 580, 268–272.
[11] Takenaka, M., Neuwirt, J. and Brennicke, A. (2004) Complex *cis*-elements determine an RNA editing site in pea mitochondria. Nucleic Acids Res. 32, 4137–4144.
[12] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.
[13] Melo-Oliveira, R., Oliveira, I.C. and Coruzzi, G.M. (1996) Arabidopsis mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc. Natl. Acad. Sci. USA 93, 4718–4723.
[14] Preiss, T., Sang, A.E., Chrzanowska-Lightowlers, Z.M.A. and Lightowlers, R.N. (1995) The mRNA-binding protein COLBP is glutamate dehydrogenase. FEBS Lett. 367, 291–296.
[15] Bringaud, F., Stripecke, R., Frech, G.C., Freedland, S., Turck, C., Byrne, E.M. and Simpson, L. (1997) Mitochondrial glutamate dehydrogenase from Leishmania tarantolae is a guide RNA-binding protein. Mol. Cell. Biol. 17, 3915–3923.
[16] Millar, A.H., Heazlewood, L., Kristensen, B.K., Braun, H.-P. and Møller, I.M. (2005) The plant mitochondrial proteome. Trends Plant Sci. 10, 36–43.